Targeting Notch to Target Cancer Stem Cells

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Abstract

The cellular heterogeneity of neoplasms has been at the center of considerable interest since the “cancer stem cell hypothesis”, originally formulated for hematologic malignancies, was extended to solid tumors. The origins of cancer “stem” cells (CSC) or tumor-initiating cells (TIC; henceforth referred to as CSCs) and the methods to identify them are hotly debated topics. Nevertheless, the existence of subpopulations of tumor cells with stem-like characteristics has significant therapeutic implications. The stem-like phenotype includes indefinite self-replication, pluripotency, and, importantly, resistance to chemotherapeutics. Thus, it is plausible that CSCs, regardless of their origin, may escape standard therapies and cause disease recurrences and/or metastasis after apparently complete remissions. Consequently, the idea of selectively targeting CSCs with novel therapeutics is gaining considerable interest. The Notch pathway is one of the most intensively studied putative therapeutic targets in CSC, and several investigational Notch inhibitors are being developed. However, successful targeting of Notch signaling in CSC will require a thorough understanding of Notch regulation and the context-dependent interactions between Notch and other therapeutically relevant pathways. Understanding these interactions will increase our ability to design rational combination regimens that are more likely to prove safe and effective. Additionally, to determine which patients are most likely to benefit from treatment with Notch-targeting therapeutics, reliable biomarkers to measure pathway activity in CSC from specific tumors will have to be identified and validated. This article summarizes the most recent developments in the field of Notch-targeted cancer therapeutics, with emphasis on CSC. Clin Cancer Res; 16(12); 3141–52. ©2010 AACR.

Cancer “Stem” Cells and Treatment Resistance

Despite decades of search for an elusive “magic bullet”, the pharmacological treatment of cancer still relies heavily on traditional chemotherapy, which is being slowly supplemented by targeted agents. Incremental improvements are being made, but treatment resistance remains a major cause of morbidity and mortality.

For patients, clinicians, and cancer biologists, the most frustrating feature of malignancies is their inherent adaptability. In the clinic, this adaptability translates into drug-resistant disease recurrence and metastasis, often after clinical and even pathological complete responses.

One possible explanation for the biological plasticity of cancers is their cellular heterogeneity. In recent years, a distinct cellular hierarchy has been identified in several hematopoietic and solid tumors. Many cancers seem to contain a small population of pluripotent “tumor initiating cells” or “cancer stem cells” (CSC; refs. 1–14). The CSC hypothesis states that CSC possess some of the biological properties of normal stem cells, including indefinite self-replication, asymmetric cell division, and resistance to toxic agents, owing, in part, to elevated expression of ABC transporters. Normal tissue stem cells are characterized by slow proliferation rates and this is generally assumed to be the case for CSC. However, it is not established that all cancer cells with stem-like markers always proliferate slowly in vivo. Additionally, CSC proliferation rates may depend on microenvironment, type of oncogenic mutations, stage of malignancy, and other variables. The origin of CSCs is the subject of considerable debate. One popular version of the “CSC hypothesis” proposes that CSC originate from the transformation of normal tissue stem cells, and give rise to other cancer cells through a
process of aberrant differentiation that resembles that of normal tissues but evades physiological regulatory mechanisms (11). CSCs from various primary tumors or cell lines do express tissue stem cells markers, including CD133 (prominin-1), nestin, c-kit, Sox2, Oct4, and musashi-1 (Fig. 1A refs. 5, 10, 15–18). On the other hand, it is also conceivable that “stemness” is a phenotype that can be acquired through transformation or modulated by extracellular signals. Stemness can be restored in normal fibroblasts by expression of Oct4, Sox2, Nanog, and LIN28 (19), or Oct3/4, Sox2, Klf4, and c-Myc (20). Physiological dedifferentiation has been described in vertebrate and invertebrate non-neoplastic systems (21).

For example, spermatogonia in the Drosophila testis can restore male germline stem cells by a Janus kinase (JAK)-signal transducer and activator of transcription (STAT)-dependent dedifferentiation process (22, 23). A well-known process of partial dedifferentiation in epithelial cancers is epithelial-mesenchymal transition (EMT; ref. 24); this consists of loss of epithelial-specific cytokeratins and E-cadherin, and acquisition of mesenchymal markers such as vimentin and N-cadherin. Transcription factors such as Twist, Snail, or Slug and transforming growth factor β (TGF-β) family secretory factors can induce EMT. A model supported by recent evidence from the Weinberg group (25) is that “stemness” can be reacquired by cancer cells when they undergo EMT, a process associated with an invasive and metastatic phenotype. An intermediate possibility between the notion that CSCs derive exclusively from normal tissue stem cells and the notion that stemness can be acquired by any cell is the suggestion that CSCs may originate from few cell populations, including tissue stem cells and immature progenitors capable of short-term self-replication. Dontu and colleagues (26) suggested that estrogen receptor (ERα)-negative breast cancers and poor-prognosis ERα-positive cancers (presumably luminal B) arise from ERα-negative primitive mammary stem cells, whereas less aggressive ERα-positive cancers (presumably luminal A) arise from ERα-positive intermediate progenitors.

These models, schematically represented in Fig. 1B, may not be mutually exclusive and may apply to different malignancies, different subtypes, or stages of the same malignancy. A common element in the different versions of the CSC hypothesis is the concept of a cellular hierarchy in solid tumors and hematological malignancy cancers similar to that of normal tissues. CSC are thought to be capable of asymmetric cell division that maintains the CSC population and produces pluripotent “progenitor-like” cells. These cells, in turn, give rise to the “bulk” tumor cells through proliferation and aberrant “differentiation” (Fig. 1C). Because of their ability to regenerate all cell types in a tumor, CSCs, and possibly progenitors, are thought to have higher tumorigenic potential than “bulk” tumor cells.

A factor that complicates the testing of this model in human tumors is the fact that the identification of CSCs, as opposed to “bulk” tumor cells in clinical specimens, relies on markers that are different for different malignancies and are not universally accepted (2, 27). A widely used functional test involves the measurement of limiting dilution tumorigenicity in immunodeficient nonobese diabetic (NOD)/severe combined immunodeficiency (SCID) mice, with CSC being highly tumorigenic in very small numbers, unlike “bulk” tumor cells. However, the tumorigenicity of human cancer cells in mice depends heavily on the degree of immunodeficiency. When melanoma cells were injected into more permissive NOD/SCID interleukin (IL)-2 receptor γ chain knockout mice (“NODG” mice) that lack natural killer (NK) cells, the fraction of tumorigenic cells seemed to be in the order of 25 to 27%, inconsistent with the rarity of tumorigenic cells identified in NOD/SCID models (28, 29). These observations indicate that the role of immune surveillance, both innate and adaptive, in defining CSCs requires careful investigation, and have led some investigators to question the CSC hypothesis. In addition to the immune system, an additional complicating factor is the role of cellular microenvironment, or CSC “niches”, where CSCs survive inside the primary tumor or at distant sites (30). Particularly in view of the fact that the stem-like phenotype may be inducible by paracrine signals such as TGF-β, Wnt, and Hedgehog, and signals transmitted by cell-cell contact such as Notch, the importance of microenvironment in determining the

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**Fig. 1.** A, schematic of an idealized CSC. The figure shows a list, not meant to be all inclusive, of pathways that modulate the CSC phenotype. CSCs exist in the context of “ niches” formed by neighboring cells and extracellular matrix (ECM). The Hedgehog (Hh), Notch, and Wnt pathways mediate short-range interactions with neighboring cells. Soluble mediators such as TGF-β and the related BMPs, or growth factors such as hepatocyte growth factor (Met ligand), as well as signals from ECM proteins may all participate in regulating the maintenance, self-renewal, and differentiation of CSCs. These are characterized by slow replication, ability to generate partially differentiated progenies (pluripotency), highly effective DNA repair, ability to eliminate xenobiotics including chemotherapeutics through ABC family transporters (ABC), and expression of primitive membrane markers (CD133, Met), Transcription factors such as Bmi-1, Musashi, Sox2, Oct4, and others are commonly expressed in putative CSCs. Immune surveillance by the innate and possibly adaptive immune systems also contributes to the CSC microenvironment, with effects that are at least in mice, are inhibitory. B, models of CSC origins. In the traditional model, CSC originate from the transformation (red dashed arrows) of normal tissue stem cells (TSC), or possibly of progenitor cells with limited self-replicability that normally generate cells destined for differentiation. In the alternative model proposed by Mani and colleagues, transformation of cells at multiple stages of the differentiation process can produce CSC through EMT, which restores a stem-like phenotype, and the ability to metastasize, to some cancer cells. Besides EMT, other mechanisms of dedifferentiation have been described and may contribute to restoration of stemness in transformed cells. C, hierarchical organization of cancers. Once CSC are formed, they are thought to generate other tumor cells through a process akin to normal tissue differentiation. In a widely accepted model, asymmetric cell division of CSCs produces pluripotent “progenitors”, which in turn generate one or more bulk tumor cell types through proliferation and aberrant differentiation. CSC and “progenitors” are more tumorigenic in xenografts and less chemosensitive than bulk cancer cells. A is adapted from Foreman et al. (14) with kind permission of Springer Science+Business Media.
biological properties of CSCs should not be underestimated. That said, clinical evidence supporting the existence of CSCs and their role in treatment resistance has emerged, particularly in breast cancer. Li and colleagues showed that tumorigenic cells with stem-like markers are selected by neo-adjuvant chemotherapy (31). Creighton and colleagues have recently shown that breast cancer cells surviving in patients after treatment with either docetaxel or letrozole have gene expression signatures characteristic of stem-like and EMT phenotypes (32). Taken together, the evidence available today suggests that cells with a stem-like phenotype are found in several human malignancies, and that, to the extent that they exist according to current hypotheses, these cells are not adequately targeted by currently used cancer therapeutics. It is reasonable to hypothesize that a complete eradication of these cells will be necessary to attain long-lasting remissions or cures. A strategy that is receiving considerable attention is to target CSC through evolutionarily ancient pathways that control self-renewal and cell fate decisions in undifferentiated, pluripotent cells (27). Many such pathways have been identified (Fig. 1A). Most of these are evolutionarily conserved and have multiple developmental roles, as well as intricate cross-talk interactions. Among them, Wnt (33), canonical Notch ligands are also transmembrane proteins -terminus. Ligand binding triggers dissociation of NEC masking an interaction between the two phobic interaction with the extracellular stump of NTM, specialized Lin-Notch repeats (LNR) that form a tight hydro-

Fig. 2). NEC contains multiple EGF-like repeats and three specialized Lin-Notch repeats (LNR) that form a tight hydrophobic interaction with the extracellular stumps of NTM, masking an "A disintegrin and metalloprotease" (ADAM) cleavage site. The region of interaction between the two subunits is called the heterodimerization domain (HD). Canonical Notch ligands are also transmembrane proteins (Fig. 2) with multiple EGF-like repeats, a short cytoplasmic tail, and a specialized delta-serrate-lag2 (DSL) domain at the N-terminus. Ligand binding triggers dissociation of N\textsuperscript{EC} from N\textsuperscript{TM}, unmasking the ADAM cleavage site (Fig. 3A, B).

\textsuperscript{N}\textsuperscript{EC} is trans-endocytosed into ligand-expressing cells whereas N is cleaved at the membrane by an ADAM, generating an intermediate called Notch extracellular truncation (N\textsuperscript{EXT}). The latter is further cleaved by \(\gamma\)-secretase, generating an active fragment (Notch intracellular; N\textsuperscript{IC}) or Notch intracellular domain (NICD; Fig. 3A). N\textsuperscript{IC} is transported into the nucleus (Fig. 3D) where it binds ubiquitination transcription factor CBF-1, Suppressor of Hairless, Lag-1 (CSL), also known as RBP-jc in mice. N\textsuperscript{IC} binds to CSL and displaces a large corepressor complex containing SKIP, SHARP, histone deacetylases, and other corepressors. The N\textsuperscript{IC}-CSL complex recruits a co-activator complex containing a Mastermind-like protein (MAML1-3 in mammals) as well as p300 and other chromatin-modifying enzymes, forming the notch transcriptional complex (NTC), and activating transcription (38). CSL target genes are numerous and include HIF1-family negative transcriptional regulators of the HES and HEY family, but also oncogenes such as c-Myc. In the nucleus, N\textsuperscript{IC} is phosphorylated by CDK8, ubiquitinated by Sel10/FWB7, and degraded by the proteasome, thus terminating the signal. Putative non-canonical pathways have been suggested but remain incompletely characterized. Among them, physical interaction of Notch-1\textsuperscript{IC} with the IKK signalosome (50), with nuclear IKK\(\alpha\) (51, 52), and with p50 (53, 54) and of Notch-3\textsuperscript{IC} with cytoplasmic IKK\(\alpha\) (55) may mediate therapeutically relevant cross-talk with nuclear factor \(\kappa\)-B (NF-\(\kappa\)B). Physical interaction of Notch-1\textsuperscript{IC} with p85 PI3-kinase \(\alpha\) (56) may mediate non-nuclear cross-talk with AKT, leading to survival signaling (57).

A closer look at this apparently simple signaling pathway reveals a dizzyingly intricate series of mechanisms that finely regulate the timing, intensity, and biological consequences of Notch signaling, and are likely to have significant therapeutic implications. The main factors that should be taken into consideration about Notch signaling in the clinical setting are listed below:

**Paralog-specific effects**

Mammals have four Notch paralogs, which differ mostly in the number of EGF repeats and the C-terminal part of N\textsuperscript{IC}. Although, in theory, they all signal through the same mechanisms, in many systems they have nonoverlapping and even opposing effects (58–61). This characteristic is relevant when comparing agents that block all Notch signaling to agents that selectively block a single receptor. In our hands, small interfering RNA (siRNA) knockdown of different Notch receptors in breast cancer cells affects the expression of different gene sets. This finding is consistent with data indicating that Notch-2, unlike Notch-1, -3, and -4, may have a positive prognostic significance in breast cancer (62, 63). Moreover, these paralog-specific effects are context-dependent, i.e., they may be different in different diseases and in CSC from different diseases. For example, in mesothelioma (64), Notch-2 inhibits the prosurvival effects of

\textit{Pannuti et al., Transcriptional profiling of Notch-target genes in breast cancer cell lines reveals paralog-specific effects, manuscript in preparation.}
Notch-1, whereas in medulloblastoma (59), Notch-2 stimulates tumorigenesis and Notch-1 inhibits it. Hence, the need to determine in CSCs from each disease and possibly disease subtype what the role of individual Notch paralogs is.

**Ligand diversity**

There are several Notch ligands with different functions. Canonical ligands include the Delta and Serrate/Jagged families (Delta-1, -3, and -4, and Jagged-1 and -2 in humans). These ligands have multiple EGF-like repeats, a DSL domain with or without a DOG domain. Serrate/Jagged family ligands also have a cysteine-rich (CR) motif (Fig. 2). It has been suggested that DSL-only ligands lacking DOG domains may function in tandem with DOG-only putative colligands such as DLK1 (reviewed in ref. 38). Noncanonical ligands such as contactins, DNER, and MAGP1-2 have been described (38). Although Notch binding to ligands expressed in trans (on contiguous cells) triggers Notch activation, some DSL-containing ligands can also bind Notch in cis (on the same cell), causing cis-inhibition. This ability is relevant to efforts to target specific ligands using monoclonal antibodies (mAb).

**Notch glycosylation**

The affinity of Notch receptors for specific ligands is controlled by NEC glycosylation. POFUT-1 decorates NEC with fucose residues, to which Fringe enzymes (in humans Lunatic, Radical, or Manic Fringe) add N-acetylglucosamine (Glc-Nac) to it. This process regulates the differential affinity of Notch receptors for their ligands. In mammals, Fringe-modified Notch-1 loses its ability to respond to Jagged ligands, whereas Notch-2 does not (38). POFUT-1 also has an unrelated chaperone activity.
that allows folded Notch to be presented at the membrane. Glucosyltransferase RUMI also modifies Notch in Drosophila (reviewed in ref. 38). Notch glycosylation has not been studied in detail in human cancers or in CSC.

**ADAM redundancy**

The ADAM cleavage step can be catalyzed by at least two proteases: ADAM17 (also known as TNF-α converting enzyme, TACE) or ADAM10. Recent evidence suggests that ADAM10 is required for ligand-dependent activation of...
Notch, whereas ADAM17 participates in ligand-independent receptor activation (65). This finding should be kept in mind when considering the use of ADAM inhibitors to block Notch activation.

Multiple γ-secretase isoforms and substrates

γ-secretase, which is targeted by several investigational drugs currently being tested in cancer, is a multisubunit transmembrane aspartyl protease that contains presenilin-1 or -2 (the catalytic subunit) plus APH1, Pen2, and Nicastrin. Because there are two presenilin isoforms and at least two APH isoforms, there can potentially exist at least four different γ-secretase complexes. There is some evidence, reviewed in (38), that these complexes have different biochemical properties and may contribute differently to Notch activation. In addition to cleaving Notch, γ-secretase catalyzes the regulated intramembranous proteolysis (RIP) of many other proteins (66). These include ADAM10 (67), putative CSC marker CD44 (66), and circulating tumor cell (CTC) marker EpCAM (68).

Multiple forms of N\textsuperscript{IC} and post-translational modifications regulating its stability

It was originally thought that γ-secretase specifically cleaves Notch before V1744. This finding led to the production of antibodies recognizing the sequence V\textsuperscript{1774}LLS, at the N-terminus of N\textsuperscript{IC}. However, γ-secretase can cleave Notch at several positions in the 1743-1748 region, with a preference for the L1746-S1747 bond (69). This finding means that there are potentially several species of N\textsuperscript{IC}, not all of which are detectable by currently available antibodies. These species have different half-lives, with the V1744 being the most stable (38). However, the intracellular stability and transcriptional activity of N\textsuperscript{IC} is regulated by many factors, including hypoxia (70, 71), prolyl isomerase Pin1 (72), and Nemo-like kinase (NLK; ref. 73). Thus, a potential role of alternative N\textsuperscript{IC}s in cancer cannot be ruled out. Cleavage site selection can be affected by the cellular compartment in which cleavage occurs (38). This is relevant to the choice of methods to detect N\textsuperscript{IC} in clinical specimens. Undetectable staining with a V\textsuperscript{1774}LLS antibody does not automatically imply lack of Notch activity.

Cellular trafficking of Notch receptors and ligands

Ubiquitination, endocytosis, and endosome sorting regulate the amount of Notch receptors at the membrane and the balance between ligand-mediated receptor activation, ligand-independent receptor activation, and degradation (37, 38). DSL-containing Notch ligands are ubiquitinated by Mindbomb or Neuralized E3 ligases, endocytosed, “activated” by a poorly understood process, and recycled to the membrane in “active” form (Fig. 3A). Upon binding, ligand-N\textsuperscript{EC} complexes are trans-endocytosed into ligand-expressing cells, a dynamin-dependent process that separates N\textsuperscript{EC} from N\textsuperscript{TM} (74). Mono-ubiquitination and endocytosis of Notch as a prerequisite to activation have been suggested (75), though this remains controversial (37, 38). The amount of Notch available at the membrane is controlled by several E3 ubiquitin ligases (Itch/AIP4, Cbl, Deltex, Nedd4), which can direct Notch to either lysosomal degradation or recycling (Fig. 3C). Deltex forms a complex with Notch and β-arrestin homolog Kurz, leading

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**Fig. 4.** Effects of a GSI on human breast cancer secondary mammospheres. Mammospheres were formed ex vivo from a pleural aspirate from a late-stage breast cancer patient. Mammospheres were dissociated and secondary mammospheres were formed and characterized for multilineage differentiation (an indication they contain pluripotent stem-like cells). Secondary mammospheres were treated with vehicle or a panel of GSIs at clinically achievable concentrations. Mammosphere and isolated cells were enumerated at different times. This example shows phase contrast microphotographs of secondary mammospheres from a clinical isolate treated with vehicle (left) or 10 μmol/L GSI MRK003 for 7 days (right). GSI completely and irreversibly blocked the formation of new mammospheres, and only isolated cells were observed in culture. These eventually underwent apoptosis.

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to Notch ubiquitination and degradation (76). In the absence of Kurz, Deltex sorts Notch into late endosomes via Rab5, causing Notch activation (77). In Drosophila, defects in endosome trafficking caused by mutations in ESCRT complex components or in tumor suppressor lethal giant disc (LGD) cause accumulation of Notch in endosomes, where Notch is activated by γ-secretase, causing aberrant cell proliferation. Aquaporin-related anion transporter big-brain (bib), which regulates endosome acidification, is required for Notch signaling (78), perhaps by facilitating the release of cleaved NIC from endosomes (38). In asymmetrically dividing cells, endocytic mediator Numb, in cooperation with ACBD3, adaptin, and Numb-associated kinase (NAK) promotes selective degradation of Notch in one of two daughter cells. This result is thought to promote differentiation of the daughter cell that loses Notch. Loss of Numb has been described in breast cancer (79) and asymmetric cell division is a hallmark of “stemness.” Loss of Numb could conceivably result in aberrant retention of “stemness.” Endocytic mediators dynamin and Rab5 are also required in Notch signal-receiving cells. Available evidence indicates that the γ-secretase cleavage step can occur at multiple cellular locations, including at or near the plasma membrane and after endocytosis (reviewed in ref. 38).

**Chromatin cross-talk**

Finally, though there are many putative CSL-responsive elements in the genome, the actual pattern of usage (and thus the gene expression changes affected by Notch activation) seem to depend on the functional interaction of Notch with other nuclear effectors, including, for example, HIF-1α (70), and the estrogen receptor ERα (52). Two NTCs can cooperatively bind to sequence-paired CSL-responsive elements (80), possibly making some promoters differentially responsive to monomeric versus dimeric NTC.

These basic observations have significant clinical implications. Simply determining levels of Notch expression at the protein or even mRNA level is not necessarily indicative of how active the pathway is in a specific cancer cell. The best target genes to use as biomarkers may well vary in different diseases and disease subsets. Investigators developing γ-secretase inhibitors (GSI) should be mindful of possible selectivity for γ-secretase isoforms, of multiple off-target effects, and of the fact that compounds with different chemical properties may affect Notch cleavage at the membrane and/or in different intracellular compartments, at neutral or acidic pH, which may directly affect the pharmacological activity of GSIs *in vivo*.

### Table 1. Notch-targeting agents

<table>
<thead>
<tr>
<th>Agent</th>
<th>Mechanism</th>
<th>Targets</th>
<th>Development Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSIs: MK0752 (Merck) R04929097 (Roche) PF-03084014 (Pfizer) LY450139 (Eli Lilly) BMS-unknown (BMS)</td>
<td>Inhibition of final Notch cleavage by γ-secretase</td>
<td>All 4 Notch paralogs; Notch ligands; multiple other γ-secretase substrates</td>
<td>Phase 1-2</td>
</tr>
<tr>
<td>GSMs: MPC-7869 (Myriad)</td>
<td>Inhibition of final substrate cleavage by γ-secretase</td>
<td>Selective for specific γ-secretase substrates</td>
<td>Unsuccessful phase 3 for MPC-7869, discovery for Notch-targeted GSMS</td>
</tr>
<tr>
<td>MAML1-stapled peptide</td>
<td>Interference with Notch nuclear co-activator MAML1</td>
<td>All four Notch paralogs, potentially other nuclear transcription factors that use MAML1</td>
<td></td>
</tr>
<tr>
<td>Notch mAbs</td>
<td>Interference with ligand-induced Notch subunit separation</td>
<td>Specific for individual Notch receptors</td>
<td>Preclinical</td>
</tr>
<tr>
<td>DLL4 mAbs</td>
<td>Interference with ligand-receptor interaction</td>
<td>Specific for Delta-4 ligand</td>
<td>Preclinical</td>
</tr>
<tr>
<td>Other ligand mAbs</td>
<td>Interference with ligand-receptor interaction</td>
<td>Specific for other Notch ligands</td>
<td>Preclinical</td>
</tr>
<tr>
<td>Notch soluble receptor decoys</td>
<td>Interference with ligand-receptor interaction</td>
<td>Relatively specific for Notch paralogs potential pan-Notch inhibition</td>
<td>Preclinical</td>
</tr>
<tr>
<td>siRNA, miRNA-based therapeutics</td>
<td>Interference with expression of Notch signaling components</td>
<td>Specific for target mRNAs</td>
<td>Preclinical</td>
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Abbreviation: microRNA (miRNA).
Targeting Notch in Cancer and CSC

Notch signaling has been implicated in a growing number of hematopoietic and solid tumors (39–49). In most cases, one or more Notch paralogs have oncogenic activity. Inappropriate Notch activation stimulates proliferation, restricts differentiation, and/or prevents apoptosis. In the skin, Notch-1 seems to act as a tumor suppressor. Recent evidence suggests that this is an indirect effect. Loss of Notch signaling in the skin causes a barrier defect that causes local inflammation, predisposing to transformation, hyperproduction of thymic stromal lymphopoietin (TSLP), and systemic immunological disturbances (81).

For a general discussion of Notch targeting in cancer, the reader is referred to (35). Here, the discussion will focus on Notch as a target in CSC.

The strongest evidence to date for a role of Notch in CSC is in breast cancer (6, 8, 82–84), embryonal brain tumors (85), and gliomas (86, 87). GSIs abolish the formation of secondary mammospheres from a variety of human breast cancer cell lines as well as primary patient specimens (Fig. 4). In breast ductal carcinoma in situ (DCIS), the ability to form multilineage spheroids ("mammospheres," an indicator of stem-like cells) is dramatically decreased by GSIs, a Notch-4 monoclonal antibody or gefitinib (83). This finding suggests cooperation between epidermal growth factor receptor (EGFR) and Notch-4 in DCIS "stem cell" maintenance. There is evidence for a feedback loop between Her2/Neu and Notch (88, 89), which may maintain CSC in Her2/Neu-overexpressing tumors (90). Sansone and colleagues (91) showed that in mammospheres from human breast cancers, IL-6 induces Notch-3 signaling, increases expression of Jagged-1, and, through Notch-3, promotes a hypoxia-resistant phenotype. The same group (84) described a p66Shc-Notch-3 pathway as essential to maintain the hypoxia-resistant phenotype of human breast cancer mammospheres. Fan and colleagues (85) showed that Notch inhibition selectively depletes medulloblastoma CSC as determined by CD133-high status or dye exclusion. The same group has described very similar findings in glioblastoma CSC (86). Importantly, in gliomas Notch seems to confer radio-resistance to CSC (87). GSI treatment selectively enhanced radiation-induced death of glioma CSC but not bulk glioma cells. This effect was replicated by Notch-1 or Notch-2 knockdown, and was accompanied by AKT inhibition and reduced and Mcl-1 expression. Other malignancies are being actively investigated. A role of Notch, STAT3 and TGF-β in hematopoietic and/or cell-mediated carcinoma CSC maintenance has been suggested (92). In gemcitabine-resistant pancreatic carcinoma cells, EMT is associated with activation of Notch signaling, potentially linking Notch to the "Weinberg model" of stemness acquisition through EMT (93) and to treatment resistance. Inhibition of Notch signaling through GSIs (86) or Delta-4 mAb (94) decreased the numbers of CSC and/or their tumorigenicity in some preclinical models.

These encouraging results suggest that therapeutic regimens including Notch inhibitors may be used in the clinic to target CSC and reverse or prevent chemotherapeutic resistance. However, for clinicians interested in targeting Notch in CSC, numerous questions remain to be addressed. There are numerous investigational Notch inhibitors to choose from, some of which are already in the clinic (Table 1). At least four chemically distinct GSIs are being developed by pharmaceutical companies. It is unclear whether these drugs are pharmacologically equivalent and, based on their evolving understanding of γ-secretase, it is possible that they may have significant differences in both Notch inhibition and off-target effects. Due to the very nature of their target, GSIs should block the cleavage of all four Notch paralogs, and multiple other γ-secretase substrates. Thus, they are relatively nonselective drugs, although in vivo their dose-limiting toxicity (secretory diarrhea) is due to Notch inhibition in intestinal stem cells. Inhibition of other γ-secretase targets may either contribute to therapeutic efficacy or hinder it, and these off-target effects may be different for different GSIs. Recently, Watters and colleagues (95) generated a library of murine mammary tumor models from genetically engineered oncogenic mammary stem cells, essentially artificial CSCs. These authors treated tumors with a specific GSI (from Merck), and describe a "GSI response signature," which is enriched for Notch pathway genes but also includes genes from G-protein-activated pathways, eicosanoid pathways, and others. Which of these effects are secondary to Notch inhibition and which are off-target remains to be established. However, these data provide a useful platform to compare different GSIs, and GSIs to other agents. The discovery that some nonsteroidal anti-inflammatory drug-related compounds can allosterically modify the substrate specificity of γ-secretase (96) suggests that Notch-selective γ-secretase modifiers (GSM) can be developed, though specificity for individual Notch homologs remains unlikely. Inhibitors of the MAML/CSL/Notch complex formation were a theoretical possibility until recently. Innovative work by Moeller and colleagues (97) shows that a hydrocarbon-stapled cell-permeable peptide derived from MAML-1 can selectively prevent the assembly of the NTC and has efficacy as a Notch inhibitor in vitro and in vivo. If nonpeptide drugs with similar characteristics can be developed, they would likely be pan-Notch inhibitors without the off-target effects of GSIs. Conversely, mAbs to Notch ligands and receptors offer single-target specificity. Antibodies that "lock" Notch receptors in an inactive conformation by preventing NkCC-N dissociation are in preclinical development (98). To date, Notch-1 mAb seem to have similar toxicities compared to GSIs. Delta-4 mAbs are effective against breast CSC in vivo (94). However, their chronic use can cause vascular neoplasms (99).

GSIs and other small molecules have the advantages of relative ease of administration, oral bioavailability, and...
low cost. Pan-Notch inhibition may or may not be an advantage depending on the relative roles of specific Notch paralogs in individual cancers, and on whether redundancy between Notch paralogs can result in resistance to single-target agents (35). Off-target effects are not necessarily a disadvantage if they contribute to efficacy. On the other hand, clinical experience so far has shown that GSI must be administered in intermittent dosing regimens to prevent dose-limiting intestinal toxicity. Whether intermittent Notch inhibition is sufficient for effective CSC targeting in patients is an open, and very important, question. If continuous inhibition is necessary for optimal results, systemically delivered GSIs may be at a disadvantage over more specific agents such as anti-ligand mAbs, or may have to be delivered selectively to tumors using innovative pharmaceutics. Situations in which a specific Notch-ligand pair is involved in CSC self-renewal may be targeted more specifically using a mAb. Tumor-selective mAbs (e.g., bispecific antibodies) would theoretically offer site-specific inhibition that could be used chronically. However, the choice of mAb for each specific indication will depend upon the Notch receptors and ligands that play a predominant role in that malignancy’s CSC.

Rational Combinations and Personalized Medicine

Even targeting developmental pathways such as Notch will most likely not give us the elusive “magic bullet”, and will require the development of rational combinations. Such combinations will be made possible only through a thorough understanding of cross-talk between Notch and other developmental and nondevelopmental pathways that may play roles in CSC in specific malignancies. Our knowledge is rapidly evolving, but there is evidence to support some combinations. The following examples are not meant to be all-inclusive, but these classes of agents are reasonable candidates for combination with Notch inhibitors: (1) Inhibitors of the PI3-kinase-AKT-mTOR pathway (35, 87, 100); (2) NF-κB inhibitors (50, 51, 101); (3) Her2/Neu inhibitors (88, 90), platinum compounds (51, 102), EGFR inhibitors (83), and Hedgehog inhibitors (103). In breast cancer, a newly discovered feedback between Notch and ERα (52, 104) supports combining Notch inhibitors with anti-estrogens. Anti-estrogen plus GSI and Hedgehog-inhibitor plus GSI combinations are being investigated in ongoing clinical trials. In the case of the Hedgehog inhibitor-GSI combination, anti-CSC effects are being measured specifically.

Ultimately, the best use of Notch inhibitors and other CSC-targeted agents will be in the context of personalized medicine. To that end, we will have to determine: (1) which cancers and specific cancer subtypes contain Notch-dependent CSC; (2) what role do specific components of Notch signaling play in these CSC; (3) what pathways cross-talk with Notch in specific CSCs; and (4) how can one measure Notch activity in CSC from individual patients (e.g., in biopsy material).

The design of clinical trials of CSC-targeted agents will have to consider that anti-CSC effects will not necessarily translate into rapid tumor volume changes. Disease-free or recurrence-free survival will be the most informative endpoints. For situations when this would require prohibitively long follow-ups, it will be important to develop accurate surrogate biomarkers that reflect anti-CSC effects. These may be spheroid formation assays, flow cytometry, molecular tests, or other tests, but post-treatment tumor tissue will be required in most cases. A question of potentially great interest is whether it is possible to assess CSC numbers or the relative "stemness" of individual tumors by studying CTCs (105–107). These cells can be isolated from patient blood by several methods, one of which is US Food and Drug Administration-approved. Although these trials may be challenging, the payoff may be novel treatments that eliminate or greatly reduce treatment resistance in a whole range of malignancies.

Disclosure of Potential Conflicts of Interest

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